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IL-1β Attenuates IFN-$\alpha\beta$-Induced Antiviral Activity and STAT1 Activation in the Liver: Involvement of Proteasome-Dependent Pathway[1]

Zhigang Tian,*† Xuening Shen,* Hong Feng,* and Bin Gao[2]*

IFN-$\alpha\beta$ is the only established treatment for viral hepatitis; however, more than 60% of patients are poorly responsive. Because viral hepatitis is associated with inflammation, we hypothesized that inflammation may attenuate the efficacy of IFN therapy. To test this hypothesis, the effect of IL-1β, one of the major proinflammatory cytokines, on IFN signaling pathway in the liver was examined. Administration of IL-1β in vivo attenuated IFN-$\alpha\beta$-induced STAT1 tyrosine phosphorylation in the liver but not in the spleen. The inhibitory action of IL-1β in vivo was not affected by depleting hepatic Kupffer cells, suggesting that IL-1β may directly target IFN-$\alpha\beta$ signaling in hepatocytes. Indeed, pretreatment of human hepatocellular carcinoma HepG2 cells with IL-1β suppressed IFN-$\alpha\beta$-induced antiviral activity and antiviral protein MxA mRNA expression. Furthermore, IL-1β attenuated IFN-$\alpha\beta$-induced STAT1 binding and tyrosine phosphorylation without affecting the level of STAT1 protein. This inhibitory effect can be reversed by pretreatment with either proteasome inhibitors or transfection of dominant negative NF-κB inducing kinase mutants. Taken together, these findings suggest that IL-1β attenuates IFN-$\alpha\beta$-induced STAT1 activation by a proteasome-dependent mechanism. In view of high levels of IL-1β in the serum or within the liver of patients with chronic liver diseases, attenuation of IFN-$\alpha\beta$ signaling in the liver by IL-1β could be one of the mechanisms underlying the resistance to IFN therapy in chronic hepatitis C, and IL-1β could be a potential therapeutic target for improving the efficacy of IFN therapy. ***The Journal of Immunology,*** **2000, 165: 3959–3965.**

Interferons are currently used as a pivotal treatment regime in a variety of virus-infected and malignant diseases, among which the liver diseases, especially the viral hepatitis and hepatocellular carcinoma, were most extensively treated by IFNs (1–5). IFN monotherapy has been used in the treatment standard for chronic hepatitis C virus infection, and has been shown to reduce serum alanine aminotransferase levels and eliminate serum virus RNA in ~40% of patients (1–3). However, more than 60% of patients are poorly responsive to IFNs (1–3), and chronic alcohol consumption further decreases the efficacy of IFN-α therapy (with <10% effectiveness in alcoholics) (6–10). The mechanisms underlying the resistance to IFN therapy are not clear, both viral and host factors have been proposed. For example, hepatitis C virus proteins (11–13) and acute ethanol exposure (14) have been shown to inhibit IFN signaling pathway. Down-regulation of IFN-$\alpha\beta$ receptor expression has been reported in the liver of viral hepatitis patients (15–17). Development of IFN Abs may be another mechanism involved in the IFN therapy resistance (18). Attempts to improve patient response to IFN therapy have included modifying the dose or dosing regimen of IFN-α, or combining IFN-α with other antiviral agents (e.g., Ribavirin) (1–3).

Clinical data indicated that a variety of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, were significantly elevated in the serum or within the liver of patients with hepatitis virus infection (19–21), liver cancer (22), or alcoholic liver diseases (23–27). We hypothesized that these elevated proinflammatory cytokines (such as IL-1β) may also regulate IFN signaling pathway and may be implicated in the IFN resistance. In attempting to understand how IL-1β modulates IFN-$\alpha\beta$ signaling pathway, both IL-1β and IFN-$\alpha\beta$ signaling pathways were briefly reviewed here. The binding of IL-1β with its receptor leads to activation of IL-1 receptor-associated kinases IRAK-1 and IRAK-2, followed by activation of NF-κB-inducing kinase (NIK)[3] and consequent IκB kinase (IκK). The activated IκK then phosphorylates the NF-κB inhibitory protein IκBα, leading to its ubiquitination and degradation through the ubiquitin-dependent proteasome pathway, followed by activation of NF-κB (reviewed in Refs. 28–31). In addition to the NF-κB signaling pathway, IL-1β also activates p42/44 mitogen-activated protein (MAP) kinase, p38 MAP kinase, c-Jun NH_2-terminal kinase, and phosphatidylinositol 3-kinase (reviewed in Refs. 28–31).

Type I (predominantly $\alpha\beta$) and type II (γ) IFNs signal through distinct but related pathways via binding to type I (IFNAR1 and IFNAR2) and type II (IFNGR1 and IFNGR2) receptors, respectively (reviewed in Refs. 32–35). Upon ligand binding, receptor-associated tyrosine kinases (Janus kinase-1 (JAK-1) and Tyk2 for type I receptor; JAK-1 and JAK-2 for type II receptor) are activated and followed by activation of STAT1. Activated STAT1 then translocates to the nucleus to activate the transcription of many target genes, including many antiviral proteins, such as the

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[3] Abbreviations used in this paper: NIK, NF-κB-inducing kinase; VSV, vesicular stomatitis virus; DMSA, DNA gel mobility shift assay; KCR, Kupffer cell receptor; JAK, Janus kinase; SOCS, suppressor of cytokine signal; Mx protein, a karyophilic 75,000-Da protein induced by IFN in mouse cells carrying the influenza virus resistance allele Mx^+.

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PKR protein kinase, MxA, and 2′-5′ oligoadenylate synthetase (reviewed in Refs. 32–35). The essential role of IFN-activated STAT1 in antiviral and anti-tumor activities is clearly demonstrated in STAT1 knockout mice (36, 37). In these mice, IFN signaling is defective and the innate response to viral or bacterial infection is absent.

Here, we demonstrated that administration of IL-1β in vivo markedly attenuated IFN-αβ-induced STAT1 tyrosine phosphorylation and hepatic Kupffer cells were not responsible for this inhibitory action. Furthermore, we have used a hepatic cell model system to explore the inhibitory effect of IL-1β on IFN-αβ signal pathway. In this system, we have demonstrated that IL-1β inhibited IFN-αβ-induced STAT1 activation and antiviral protein expression. The underlying mechanisms by which IL-1β modulates IFN-αβ signaling pathway were also explored.

Materials and Methods

Materials

Female ICR mice (15–20 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). STAT1 Ab was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine-STAT1 (Tyr^{701}) Ab was obtained from Bio-Lab (Beverly, MA). The following reagents were obtained from Sigma (St. Louis, MO): IL-1β, collagenase type I and IV, sodium vanadate, and Nonidet P-40. MG132 and lactacystin were from Calbiochem (San Diego, CA). IFN-α, IFN-β, and IFN-γ were purchased from Bioscience International (Camarillo, CA). Radiolabeled [γ-^{32}P]ATP was obtained from DuPont/NEN (Boston, MA).

Antiviral assay

HepG2 cells (American Type Culture Collection, Manassas, VA) were treated with various concentrations of IL-1β for 30 min, followed by incubation with IFN-α or IFN-β for 16 h. Various concentrations of vesicular stomatitis virus (VSV) were added and incubated for 48 h. Viable cells were measured by a methylthiotetrazole assay (38).

DNA gel mobility shift assay (DMSA)

DMSA were performed in 20-μl volumes with 20 mM Tris-HCl, pH 7.9, 1.5% glycerol, 50 μg/ml BSA, 1 mM DTT, 0.5 mM PMSF, 2 μg of poly(dI-dC), 1 ng of ^{32}P-labeled probe, and 10 μg of nuclear extract. Reactions were incubated at 25°C for 20 min and subsequently analyzed by electrophoresis through nondenaturing stock 4% and 10% polyacrylamide gels in 0.5× TBE buffer containing 44.5 mM Tris-HCl, pH 8.2, 44.5 mM boric acid, and 1 mM EDTA. After prerunning the gel at 100 volts for 2 h, electrophoresis was performed at 270 V for 2 h at 4°C. The gels were exposed to PhosphorImager Exposure Cassette and analyzed by PhosphorImager ImageQuant program (Molecular Dynamics, Sunnyvale, CA). The DMSA for STAT1 binding was performed as described previously (39). The STAT-binding site in the double-stranded oligonucleotide m67 (the high affinity serum-induced element) consisting of 5′-GTC GAC ATT TCC CGT AAA TCG TCG A-3′was used as probes to determine Stat1/Stat3 binding.

RT-PCR

The RT-PCR was conducted as described previously (40). The following primer pairs were used: forward primer (5′-GCT ACA CAC CGT GAC GGA TAT GG-3′) and reverse primer (5′-CGA GCT GGA TTG GAA AGC CC-3′) for human MxA (41); the PCR product for MxA is 289 bp; forward primer (5′-TCT GTA CGT CCC AGG GAG CGG CAC C-3′) and reverse primer (5′-CTC TCC GTT CCT ATG TCT CCA-3′) for mouse Kupffer cell receptor (KCR) (PCR product: 221 bp); forward (5′-AAG ACA TCA GCC GGG CCG ACT A-3′) and reverse (5′-GTC TTG TTG GTA AAG GTA GTC-3′) for human suppressor of cytokine signal 2 (SOCS2) (PCR product: 300 bp); forward (5′-GGA CCA GCG CCA CTT CTT CAC-3′) and reverse (5′-TAC TGG TCC AGG AAC TCC CGA-3′) for human SOCS3 (PCR product: 450 bp); forward primer (5′-GTG GGG CGC CCC AGG CAC CA-3′) and reverse primer (5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′) for human β-actin.

Isolation of rat Kupffer cells

Rat hepatic Kupffer cells were isolated exactly as described previously (42). The viability and purity of the cells were constantly higher 90% as tested by trypan blue exclusion and by Kaplows nonspecific esterase staining, respectively.

Western blot analysis

Cells were resuspended in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, 1% Nonidet P-40, 10% glycerol) and then centrifuged for 10 min at 4°C. Protein concentration of the supernatant (protein fraction) was calculated using the Bio-Rad protein assay. An aliquot of 40 μg of protein was mixed with an equivalent volume of 2× protein loading buffer containing 2-ME and boiled for 5 min before loading onto an SDS/8% polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary Abs. Membranes were washed with TPBS (0.05% (v/v) Tween 20 in PBS (pH 7.4)) and incubated with a 1:4000 dilution of HRP-conjugated secondary Abs for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

JAK assay

To assess JAK phosphorylation, cells were washed twice with PBS (pH 7.4) containing 1 mM sodium vanadate and lysed in 0.5 ml of lysis buffer. The total cell extracts were immunoprecipitated with anti-JAK1 or anti-TYK2 Abs, washed twice with lysis buffer, and then once with kinase buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1 mM sodium orthovandate). Pellets were resuspended in 50 μl of kinase buffer containing 5 μCi of [γ^{32}P]ATP and incubated at 30°C for 10 min. Beads were washed twice with 500 μl of stop buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA), and then boiled in SDS sample buffer containing 2.5% 2-ME for 5 min. The solubilized proteins were resolved by SDS-PAGE and quantified by phosphorimaging.

Expression of dominant-negative mutants

The dominant-negative mutants of NIK were transfected into the cells by an adenovirus-lysine-mediated procedure as described previously (43, 44). This method can achieve 80% transfection efficiency. Briefly, adenovirus-DNA complexes were prepared by incubating lysine-modified adenovirus with dominant-negative mutants of NIK for 30 min at 25°C in the dark, followed by a 30-min incubation with polylysine at a molar concentration equivalent to 125 times the molar plasmid DNA concentrations. Adenovirus-DNA-lysine complex was then added to the cells, and incubated for 8 h at 37°C. The cells were washed with media to remove virus and cultured for an additional 48 h in DMEM containing 10% FCS. The dominant-negative mutants of NIK (KK429-430AA) were generous gifts from Dr. David Wallach (Weizmann Institute of Science, Rehovot, Israel).

Results

IL-1β suppresses IFN-α-activated STAT1in the liver but not in the spleen in vivo

As mentioned in the introduction, the levels of IL-1β were elevated in the serum or within the liver of patients with a variety of chronic liver diseases (19–27). To test whether the elevated IL-1β modulates the efficacy of IFN therapy, the effects of IL-1β on IFN-αβ-activated STAT1 in vivo were examined. As shown in Fig. 1*A*, injection of IFN-α or IFN-β was able to induce STAT1 tyrosine phosphorylation in the liver in a dose-dependent manner, with evident at 40 μg/kg body weight for both IFN-α and IFN-β. These findings indicate that IFN-αβ is able to induce STAT1 tyrosine phosphorylation in the liver in vivo. Next, the effect of IL-1β on IFN-αβ activation of STAT1 in the liver in vivo were examined. As shown in Fig. 1*B*, injection of IL-1β for 30 min or 1 h markedly attenuated IFN-α- but only slightly inhibited IFN-γ-induced STAT1 tyrosine phosphorylation in the liver (*lanes 3* and *4* vs *lane 2*). Interestingly, the same IL-1β treatment did not affect IFN-α- or IFN-γ-activated STAT1 in the spleen (Fig. 1*B*). The unchanged STAT1 density in Fig. 1*B* indicated that treatment with IL-1β and/or IFN-α or IFN-γ did not affect the levels of STAT1 protein expression in the liver or spleen. These findings suggest that IL-1β is able to suppress IFN-α signaling pathway in the liver in vivo.

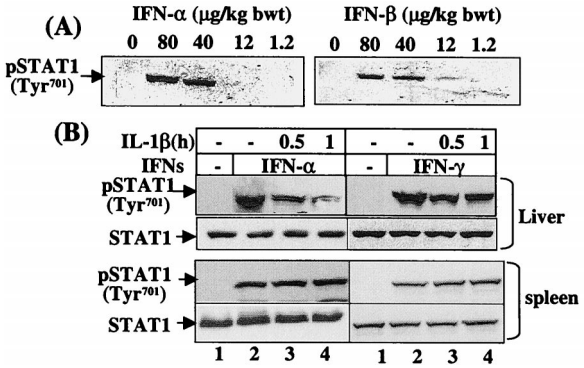

FIGURE 1. IL-1β inhibits IFN-$\alpha\beta$- but not IFN-γ-activated STAT1 in the liver in vivo. *A*, ICR mice were administrated i.v. with various concentrations of IFN-α or IFN-β. After 30 min, liver extracts were subjected to Western blotting using an anti-phospho-STAT1 (Tyr^{701}) Ab. *B*, ICR mice were administrated (i.v.) with IL-1β (40 μg/kg body weight) for 30 min or 1 h, followed by injection (i.v.) with IFN-α (40 μg/kg body weight) or IFN-β (40 μg/kg body weight). After 30 min, liver extracts or spleen extracts were subjected to Western blotting using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 Abs. Blots shown are representative of three independent experiments.

Kupffer cells are not the major cells responsible for IL-1β suppression of IFN-α-activated STAT1 in the liver in vivo

It has been shown that Kupffer cells play a key role in hepatic inflammation response (45). We wondered whether they were also involved in IL-1β suppression of IFN-activated STAT1 in vivo. To test this hypothesis, gadolinium chloride ($GdCl_3$), which has been widely used to deplete Kupffer cells (46, 47), was used. To ensure that $GdCl_3$ treatment effectively depleted the hepatic Kupffer cells, expression of KCR (also called carbohydrate-binding receptor), a gene unique to Kupffer cells (48), was measured by RT-PCR. As shown in Fig. 2*A*, after treatment with $GdCl_3$ for 24 or 48 h, expression of KCR in the liver was markedly decreased. This result indicated that treatment with $GdCl_3$ (10 mg/kg body weight) for 24 h or 48 h was able to deplete the hepatic Kupffer cells. Next, we examined whether depletion of hepatic Kupffer cells could modulate the inhibitory effect of IL-1β on IFN-α-activated STAT1. As shown in Fig. 2, *B* and *C*, depletion of Kupffer cells slightly enhanced IFN-α-activated STAT1 in the liver (Fig. 2*B*, *lane 3* vs *lane 2*) but did not antagonize the inhibitory action of IL-1β on IFN-α signaling in the liver (Fig. 2*C*, *lane 4* vs *lane 3*). These findings suggest that IL-1β suppression of IFN-α-activated STAT1 in the liver does not require Kupffer cells but this does not prove that Kupffer cells are not targeted. To test whether Kupffer cells are targeted, Kupffer cells were isolated and treated with IL-1β and/or IFN-α. As shown in Fig. 2*D*, IFN-α activated STAT1 in Kupffer cells, pretreatment of cells with IL-1β attenuated IFN-α-activated STAT1. Taken together, these findings indicate that Kupffer cells are targeted by IFN-α but are not the major cells responsible for IFN-α-activated STAT1 in the liver in vivo, and suggest that IL-1β may target directly IFN-α signaling in hepatocytes. Thus, effects of IL-1β on IFN-$\alpha\beta$-induced antiviral activity, antiviral protein expression, and signaling transduction in hepatocytes were examined (see below).

IL-1β inhibits the antiviral effect of IFN-$\alpha\beta$ in human hepatocellular carcinoma HepG2 cells

To test whether IL-1β inhibited the antiviral activity of IFNs, the VSV was used because this virus has been shown to infect HepG2 cells and is an effective target for IFN treatment (49–51). As shown in Fig. 3, after 2 days, VSV was able to infect and destroy the HepG2 cells (about 65% of cell death). Treatment of cells with IFN-α or IFN-β significantly inhibited VSV-induced cell death (there was only 35% of cell death in this group). IL-1β alone did not show any antiviral effect, but it markedly counteracted the antiviral activity of IFN-α or IFN-β in a concentration-dependent manner (from 0.3 to 20 ng/ml). IFN treatment usually provides several log protection against VSV infection (50, 51), whereas we only observed partial inhibition of VSV infection in Fig. 3. This is probably due to lack of several antiviral proteins in HepG2 cells (52). These findings indicate that IL-1β is able to antagonize IFN-$\alpha\beta$ antiviral function in HepG2 cells, which is in agreement with two previous reports showing that IL-1β suppressed the antiviral activity of IFN-$\alpha\beta$ in monkey hepatic parenchymal cells (50) and human FS-fibroblasts (51).

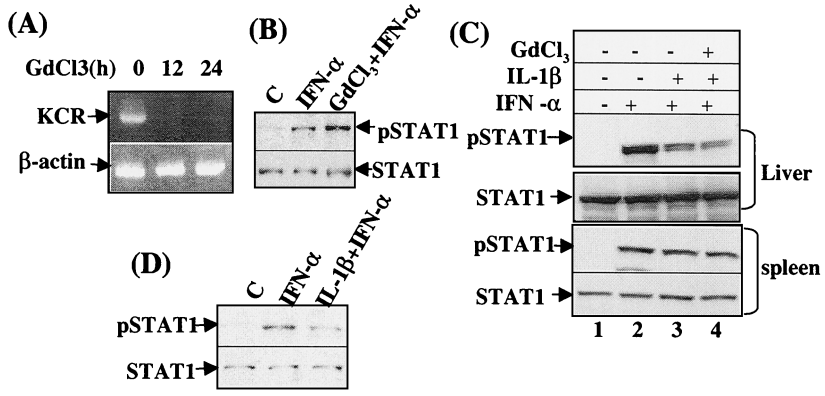

FIGURE 2. Kupffer cells are not involved in IL-1β attenuation of IFN-α-activated STAT1 in the liver. *A*, ICR mice were injected i.v. with $GdCl_3$ (10 mg/kg body weight) for 24 h or 48 h. Total RNA was isolated from the liver and subjected to RT-PCR by using KCR primers as described in *Materials and Methods*. *B*, ICR mice were injected i.v. with saline or $GdCl_3$ for 24 h, followed by injection (i.v) with IFN-α for 30 min. Liver extracts were subjected to Western blot analysis using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 Abs. *C*, ICR mice were administrated (i.v.) with gadolinium chloride ($GdCl_3$, 10 mg/kg body weight) for 24 h, followed by injection of IL-1β (40 μg/kg body weight) for 30 min, and consequent injection (i.v.) with IFN-α (40 μg/kg body weight). After 30 min, liver extracts or spleen extracts were subjected to Western blotting using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 Abs. *D*, Kupffer cells were treated with IL-1β for 60 min, followed by simulation with IFN-α (250 U/ml) for 30 min. Cell extracts were subjected to Western blot analysis using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 Abs. Blots shown are representative of three independent experiments.

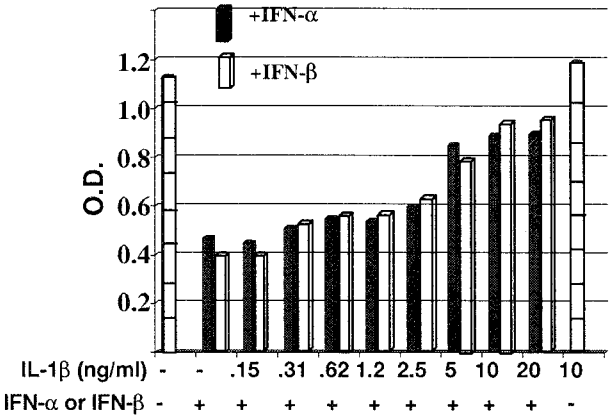

FIGURE 3. IL-1β inhibits the antiviral activity of IFN-αβ in HepG2 cells. HepG2 cells were treated with or without IL-1β (from 0.15 to 20 ng/ml) for 1 h, and then treated with IFN-α (250 U/ml) or IFN-β (250 U/ml) for another 4 h, followed by infection with VSV virus. Two days later, the cell activities were tested by MTT method, as described in *Materials and Methods*. This experiment was repeated three times and similar results were obtained.

Effects of IL-1β on IFN-αβ-induced expression of antiviral proteins

To define the mechanism by which IL-1β inhibits IFN-αβ-mediated antiviral activity, we examined the effects of IL-1β on IFN-αβ-induced antiviral protein expression. Because the MxA is the most sensitive and extensively studied antiviral protein (32–35), the effect of IL-1β on IFN-αβ-induced MxA was examined. As shown in Fig. 4, treatment of HepG2 cells with IFN-α or IFN-β for 4 h caused a significant increase of MxA gene expression (*lanes 7* and *3*, respectively). IL-1β alone did not affect MxA gene expression (*lane 2*) but markedly attenuated IFN-α or IFN-β-induced MxA gene expression (*lane 10* vs *lane 7*; *lanes 4–6* vs *lane 3*), with most evident after 60 min of treatment. The unchanged β-actin in the bottom panel indicated that IL-1β suppression of IFN-αβ-induced MxA expression was real and not the result of uneven loading. These findings indicate that IL-1β inhibits IFN-αβ-induced antiviral protein MxA gene expression, which may be implicated in IL-1β suppression of IFN-αβ-mediated antiviral activity.

IL-1β inhibits IFN-αβ-induced STAT1 binding and tyrosine phosphorylation without affecting the activation of JAK1 and TYK2

To further examine the molecular mechanism by which IL-1β attenuates IFN-αβ-induced MxA gene expression, the effect of

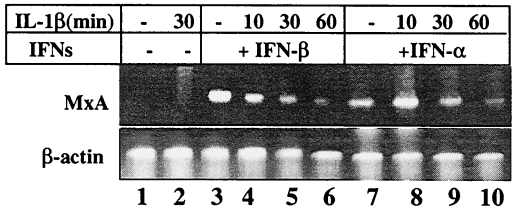

FIGURE 4. IL-1β inhibits IFN-αβ-induced MxA gene expression. HepG2 cells were treated with IL-1β (10 ng/ml) for various time periods as indicated, followed by stimulation with IFN-α (250 U/ml) or IFN-β (250 U/ml) for another 4 h. Total RNA was then prepared and subjected to RT-PCR by using MxA or β actin primers as described in *Materials and Methods*. Autoradiograms or blots shown are representative of two independent experiments.

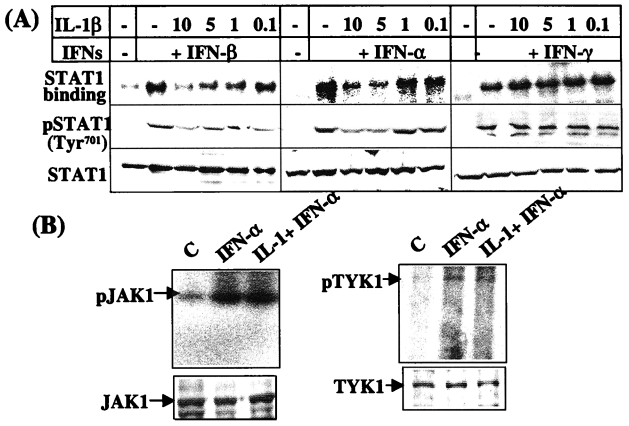

FIGURE 5. IL-1β inhibits type I IFN (IFN-α and IFN-β) but not type II IFN (IFN-γ)-activated STAT1 without affecting the activation of JAK1 and TYK1. *A*, HepG2 cells were treated with various concentrations of IL-1β (0.1–10 ng/ml) for 60 min, followed by a 30-min stimulation with IFN-α (250 U/ml), IFN-β (250 U/ml), or IFN-γ (10 ng/ml). Cell extracts were then prepared and subjected to DMSA using the oligonucleotide m67 as a probe, or Western blotting using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 Abs. *B*, HepG2 cells were treated with IL-1β (10 ng/ml) for 60 min, followed by a 5-min stimulation with IFN-α. Cell extracts were prepared and subjected to JAK1 and TYK2 kinase assays as described in *Materials and Methods*. Autoradiograms or blots shown are representative of three independent experiments.

IL-1β on IFN-αβ signaling pathway was investigated. As shown in Fig. 5*A*, pretreatment of HepG2 cells with IL-1β attenuated IFN-α- or IFN-β-induced STAT1 binding and tyrosine phosphorylation (Tyr^{701}) in a concentration-dependent manner. IL-1β inhibition of IFN-β was evident at 1 ng/ml and inhibition of IFN-α was evident at 5 ng/ml. On the contrary, the same IL-1β treatment did not affect IFN-γ-induced STAT1 binding and phosphorylation. These findings indicate that IL-1β inhibits type I IFN (IFN-αβ)- but not type II IFN (IFN-γ)-induced STAT1 binding and tyrosine phosphorylation in HepG2 cells. The same density of unphosphorylated STAT1 in the bottom panel indicated that treatment with IL-1β and/or IFN-α,- β, -γ did not affect the levels of STAT1 protein expression.

To define whether IL-1β suppression of IFN-αβ signaling is due to inactivation of the upstream activators, the effect of IL-1β on JAK1 and TYK2 was examined. As shown in Fig. 5*B*, pretreatment of HepG2 cells with IL-1β for 60 min did not significantly inhibit IFN-α-induced JAK1 or TYK2 kinase activation. These findings suggest that IL-1β inhibits IFN-αβ-induced STAT1 binding and tyrosine phosphorylation without affecting the activation of JAK1 and TYK2.

Evidence for the involvement of proteasome-dependent mechanism in the inhibitory action of IL-1β on IFN-αβ-activated STAT1

The binding of IL-1β with its receptor leads to IκBα ubiquitination and degradation through the ubiquitin-dependent proteasome pathway, followed by activation of NF-κB (28–32). This pathway has been implicated in down-regulation of the JAK-STAT signaling pathway (53). Therefore, we hypothesized that the proteasome-dependent pathway may also be involved in IL-1β attenuation of IFN-α-activated STAT1 in the liver. To test this hypothesis, two highly specific proteasome inhibitors MG132 and lactacystin were used. As shown in Fig. 6, both inhibitors attenuated IL-1β-induced NF-κB activation (Fig. 6*A*) and markedly antagonized the inhibitory effect of IL-1 on IFN-αβ-induced STAT1 binding and tyrosine phosphorylation (Fig. 6*B*, *lane 4* vs *lane 2*). The bottom

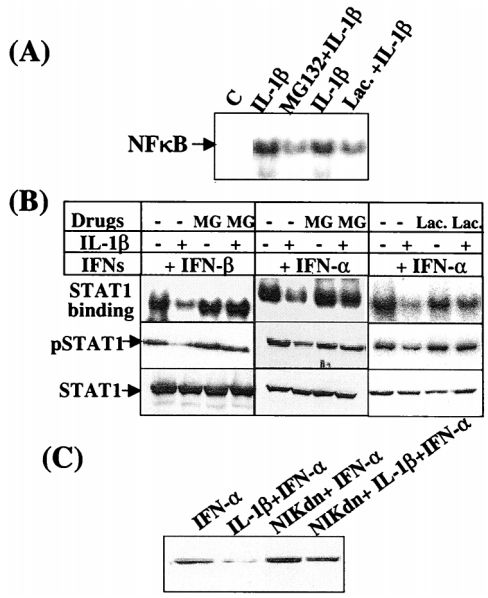

FIGURE 6. Evidence for the involvement of the proteasome-dependent pathway in IL-1β inhibition of IFN-$\alpha\beta$-activated STAT1 in HepG2 cells. *A*, HepG2 cells were treated with MG132 (50 μM) or lactacystin (50 μM) for 30 min, followed by a 60-min stimulation with IL-1β (20 ng/ml). Cell extracts were then subjected to gel mobility shift assay using NFκ-B oligonucleotide probe. *B*, HepG2 cells were treated with MG132 (50 μM) or lactacystin (50 μM) for 30 min, then incubated with IL-1β (10 ng/ml) for 60 min, followed by a 30-min stimulation with IFN-α (250 U/ml) or IFN-β (250 U/ml). Cell extracts were then subjected to DMSA using the oligonucleotide m67 as a probe, or Western blotting using anti-phospho- STAT1 (Tyr^{701}) or anti-STAT1 Abs. *C*, HepG2 cells were transfected with dominant-negative NIK mutants (NIKdn) for 48 h, followed by a 60-min stimulation with IL-1β (20 ng/ml) and a 30-min stimulation with IFN-α (250 U/ml). Cell extracts were then subjected to Western blot analysis using anti-phospho-STAT1 (Tyr^{701}) Ab. Autoradiograms or blots shown are representative of three independent experiments.

panel in Fig. 6*B* indicated that these treatments did not affect the levels of STAT1 protein expression. These findings suggest that the proteasome-dependent proteolytic mechanism is involved in both IL-1β activation of NF-κB and dephosphorylation of IFN-$\alpha\beta$-activated STAT1.

To further confirm the involvement of NIK-driven proteasome pathway in IL-1β suppression of IFN-α-activated STAT1, NIK (KK429-430AA) dominant negative mutants were used. NIK (KK429-430AA) DNA was transfected by an adenovirus-lysine-mediated procedure. This method can achieve 80% transfection efficiency (43). As shown in Fig. 6*C*, transfection with dominant negative NIK mutants antagonized IL-1β inhibition of IFN-α-activated STAT1 (*lane 4* vs *lane 2*). These findings suggest that NIK is involved in IL-1β suppression of IFN-α-activated STAT1.

Rapid inhibition of IFN-α-activated STAT1 by IL-1β does not require new protein synthesis

It has been reported that LPS and bacteria inhibited the JAK-STAT signaling pathway through induction of SOCS (54) and IL-1β attenuated growth hormone-activated STAT5 through induction of SOCS3 in rat liver H4-II-E cells (55). We wondered whether induction of SOCS protein was also involved in IL-1β suppression of IFN-α-activated STAT1 in HepG2 cells. As shown in Fig. 7*A*, treatment of HepG2 cells with IL-1β for 30 min markedly inhibited IFN-α-activated STAT1, whereas treatment for 12 h or 24 h had no effects. This suggests that IL-1β suppression of IFN-α-activated STAT1 in HepG2 cells is rapid. Next, we asked whether such rapid inhibition required new protein synthesis. As shown in Fig. 7*B*, blocking new protein synthesis with cyclohexmide did not abolish IL-1β suppression of IFN-α-activated STAT1, suggesting that rapid inhibition of IFN-α-activated STAT1 by IL-1β does not require new protein synthesis. Moreover, IL-1β did not significantly induce expression of SOCS2 and SOCS3 in HepG2 cells (Fig. 7*C*). Taken together, these findings suggest that IL-1β suppression of IFN-α-activated STAT1 in HepG2 cells is rapid and does not require new protein (i.e., SOCS) synthesis.

Discussion

It has been reported that IL-1 treatment in vitro attenuated the antiviral activity of IFN-$\alpha\beta$ in monkey hepatic parenchymal cells (50) and human FS-fibroblasts (51), however, the underlying mechanism remains unknown. Here we demonstrated for the first time that IL-1β antagonized the antiviral activity of IFN-$\alpha\beta$ in human hepatocellular carcinoma HepG2. Additional experiments suggest that the inhibitory action of IL-1β on the antiviral activity of IFN-$\alpha\beta$ is probably due to down-regulation of antiviral protein expression and inhibition of IFN-$\alpha\beta$-activated STAT1. Moreover,

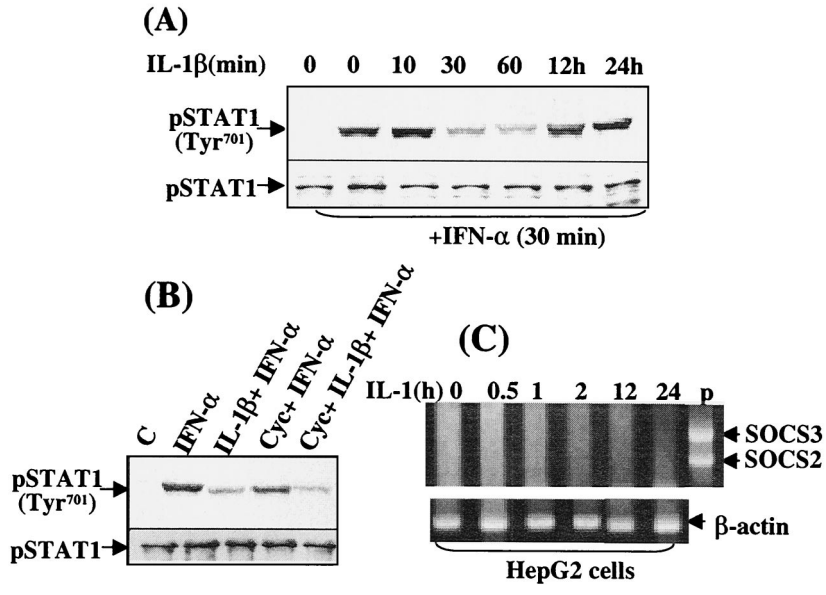

FIGURE 7. Rapid inhibition of IFN-α-activated STAT1 by IL-1β does not require new protein synthesis. *A*, HepG2 cells were treated with IL-1β for various time periods. Cell extracts were then subjected to Western blot analysis using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 Abs. *B*, HepG2 cells were incubated with cyclohexmide (Cyc, 20 μg/ml) for 30 min, then treated with IL-1β (10 ng/ml) for 60 min, followed by a 30-min stimulation with IFN-α (250 U/ml). Cell extracts were then subjected to Western blot analysis using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 Abs. *C*, HepG2 cells were treated with IL-1β (10 ng/ml) for various time points as indicated. Total RNA was then prepared and subjected to RT-PCR using SOCS2 or SOCS3 primer pairs as described in *Materials and Methods*. P, Positive control; SOCS2 and SOCS3 cDNAs were used as a template. Autoradiograms or blots shown are representative of two independent experiments.

administration of IL-1β in vivo markedly attenuated IFN-αβ-activated STAT1 in the liver, suggesting that IL-1β may also inhibit the antiviral activity of IFN-αβ in vivo.

The molecular mechanism by which IL-1β attenuates IFN-αβ-activated STAT1 in the liver was also explored in this paper. It is well established that the binding of IL-1β with its receptor leads NIK activation and consequently degrades IκB through the ubiquitin-dependent proteasome pathway, followed by activation of NF-κB (28–31). Here we demonstrated that blocking NIK (Fig. 7) or proteasome pathway (Fig. 6) abolished both IL-1β activation of NF-κB and IL-1β suppression of IFN-αβ-activated STAT1, suggesting that an analogous mechanism may be involved in both IL-1β activation of NF-κB and suppression of IFN-αβ-activated STAT1. To best interpret these findings, we proposed a model (summarized in Fig. 8) that allows for cross-talk between IL-1β and IFN-αβ signaling pathways and consequent inhibition of IFN-αβ-induced antiviral activity. In this model, IL-1β stimulates NF-κB-dependent gene expression, whereas it concomitantly suppresses IFN-αβ-induced gene expression. IL-1 activates a series of kinases including NIK that cause degradation of IκB through the ubiquitin-proteasome pathway (33–35). This pathway may simultaneously degrade a putative an unknown protein, followed by releasing an inhibitory factor that attenuates STAT1 tyrosine phosphorylation and so blocks STAT1-dependent activation of transcription, including MxA gene expression. The identity of these unknown proteins requires further studies.

The proteasome-dependent pathway for protein degradation has been implicated in down-regulation of STAT activation induced by several other cytokines (besides IL-1 and IFN). For example, this pathway has been implicated in down-regulation of STAT activation induced by IFN-γ (53), IL-2 (56), IL-3 (57), and growth hormone (58). These findings led us to speculate that IL-β may also suppress the activation of STAT signaling induced by these cytokines. However, the data in Fig. 5 showed that IL-1β did not attenuate IFN-γ-activated STAT1, suggesting that IL-1β does not target STAT1 in general and may target directly IFN-αβ receptors. The mechanism by which IL-β attenuates IFN-αβ receptors requires further investigation.

Induction of SOCS proteins is an important mechanism responsible for down-regulation of the JAK-STAT signaling pathway (59). SOCS proteins are relatively small proteins that contain a central SH2 domain and a conserved C-terminal SOCS box. It is believed that SOCS attenuated the JAK-STAT signaling pathway through binding to the phosphorylated tyrosine residues on JAK proteins (59). It has been shown that IL-1β can induce SOCS3 gene expression in H4-II-E cells and consequently attenuate growth hormone-activated STAT5 (55). However, three lines of evidence suggest that SOCS is not involved in IL-1β suppression of IFN-α-activated STAT1 in HepG2 cells. First, the inhibitory action of IL-1β is rapid with evident inhibition after 30 min stimulation (Figs. 5*A* and 7*A*). Second, SOCS is an inducible protein whereas the inhibitory action of IL-1β does not require new protein synthesis (Fig. 7). Third, treatment of HepG2 cells for various time periods with IL-1β did not significantly induce SOCS2 and SOCS3 gene expression (Fig. 7*C*).

In present paper, we also demonstrated for the first time that IL-1β inhibits IFN-α-activated STAT1 in the liver (Fig. 1). These findings suggest that IL-1β may attenuate the antiviral action of IFN-α in the liver in vivo. Although Kupffer cells are targeted by both IL-1β and IFN-α (Figs. 2, *B* and *D*), depletion of these cells by injection of $GdCl_3$ slightly potentiated IFN-α-activated STAT1 in the liver (Fig. 2*B*). This suggests that Kupffer cells are not the major cells responsible for IFN-α-induced STAT1 activation in vivo. Furthermore, depletion of Kupffer cells did not antagonize IL-1β suppression of IFN-αβ-activated STAT1 in the liver in vivo, indicating that Kupffer cells are not involved and IL-1β may directly target IFN-αβ signaling in hepatocytes in vivo. The latter is consistent with the in vitro data that showed IL-1β suppression of IFN-αβ-activated STAT1 in hepatic cells (Figs. 5–7).

In summary, in the present report we provide the first evidence that IL-1β, one of major proinflammatory cytokines, inhibits IFN-αβ-activated STAT1 in the liver in vivo and attenuates IFN-αβ-activated STAT1, antiviral activity, and antiviral protein expression in HepG2 cells. It has been reported that the levels of IL-1β in the serum or within the liver were markedly elevated in the patients with chronic liver diseases (19–27), therefore, IL-1β attenuation of IFN-αβ signaling in the liver could be one of mechanisms underlying the IFN therapy resistance in chronic hepatitis C, and IL-1β could be a potential therapeutic target for improving the efficacy of IFN therapy.

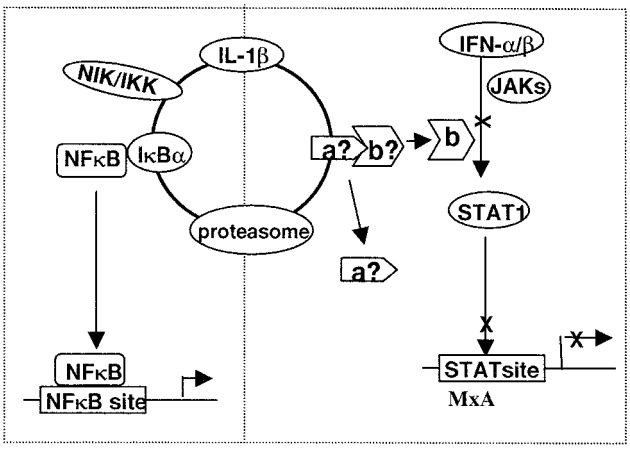

FIGURE 8. Working hypothesis for molecular mechanism by which IL-1β inhibits IFN-αβ-activated STAT1. For IL-1β-dependent gene expression (depicted in the left), receptor binding of IL-1β leads to activation of NIK and IκB kinase (IκK), followed by phosphorylation and degradation of the NF-κB inhibitory protein through the ubiquitin-dependent proteasome pathway. Degradation of IκB allows NF-κB to translocate to the nucleus and mediate transcription of appropriate target genes containing the NF-κB binding site. For IFN-αβ-dependent gene expression (depicted in the right), IFN-αβ binds to type I receptor and activates JAKs and STAT1. Phosphorylated STAT1 translocates to nucleus and activates the transcription of antiviral protein, such as MxA. For IL-1β-dependent inhibition of IFN-αβ-activated STAT1, IL-1β may similarly act to promote proteasome-dependent degradation of an unknown protein (a?), followed by releasing an inhibitory protein (b?, possibly a PTP), which may promote STAT1 dephosphorylation.

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References

1. Lindsay, K. L. 1997. Therapy of hepatitis C: overview. *Hepatology 26(Suppl. 1):71S.*
2. Gish, R. G. 1999. Standards of treatment in chronic hepatitis C. *Semin. Liver Dis. 19(Suppl. 1):35.*
3. Davis, G. L. 1999. Combination therapy with interferon α and Ribavirin as retreatment of interferon relapse in chronic hepatitis C. *Semin. Liver Dis. 19(Suppl. 1):49.*
4. Musch, E., B. Hogemann, A. Gerritzen, H. Fischer, M. Wiese, W. Kruis, M. Malek, R. Gugler, G. Schmidt, H. Huchzermeyer, et al. 1998. Phase II clinical trial of combined natural interferon-32 β plus recombinant interferon-γ treatment of chronic hepatitis B. *Hepatogastroenterology 45:2282.*

5. Horiike, N., T. Fujisawa, K. Michitaka, K. Tada, T. Masumoto, H. Iuchi, N. Kojima, and M. Onji. 1998. The effectiveness of interferon therapy on occurrence of hepatocellular carcinoma in chronic hepatitis C. *Oncol. Rep. 5:1171.*
6. Schiff, E. R. 1997. Hepatitis C and alcohol. *Hepatology 26:39S.*
7. Ohnishi, K., S. Matsuo, K. Matsutani, M. Itahashi, K. Kakihara, K. Suzuki, S. Ito, and K. Fujiwara. 1996. Interferon therapy for chronic hepatitis C in habitual drinkers: comparison with chronic hepatitis C in infrequent drinkers. *Am. J. Gastroenterol. 91:1374.*
8. Okazaki, T., H. Yoshihara, K. Suzuki, Y. Yamada, T. Tsujimura, K. Kawano, Y. Yamada, and H. Abe. 1994. Efficacy of interferon therapy in patients with chronic hepatitis C: comparison between non-drinkers and drinkers. *Scand. J. Gastroenterol. 29:1039.*
9. Oshita, M., N. Hayashi, A. Kasahara, H. Hagiwara, E. Mita, M. Naito, K. Katayama, H. Fusamoto, and T. Kamada. 1994. Increased serum hepatitis C virus RNA levels among alcoholic patients with chronic hepatitis C. *Hepatology 20:1115.*
10. Lawrence, S. P. 2000. Advances in the treatment of hepatitis C. *Adv. Intern. Med. 45:65.*
11. Gale, M., Jr., and M. G. Katze. 1998. Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase. *Pharmacol. Ther. 78:29.*
12. Heim, M. H., D. Moradpour, and H. E. Blum. 1999 Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *J. Virol. 73:8469.*
13. Taylor, D. R., S. T. Shi, P. R. Romano, G. N. Barber, and M. M. Lai. 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science 285:107.*
14. Nguyen, V. T., F. Hong, E. J. Ishac, J. Chen, and B. Gao. 2000. Interferons activate p42/44 MAP kinase and JAK-STAT signaling pathways in hepatocytes: differential regulation by acute ethanol via a PKC-dependent mechanism. *Biochem. J. 349:427.*
15. Fukuda, R., N. Ishimura, Y. Kushiyama, N. Moriyama, S. Ishihara, S. Nagasawa, T. Miyake, M. Niigaki, S. Satoh, S. Sakai, et al. 1997. Effectiveness of interferon-α therapy in chronic hepatitis C is associated with the amount of interferon-α receptor mRNA in the liver. *J. Hepatol. 26:455.*
16. Yatsuhshi, H., K. Yamasaki, T. Aritomi, P. Carmen, O. Inoue, M. Kiga, and M. Yano. 1999. Quantitative analysis of interferon $\alpha\beta$ receptor mRNA in the liver of patients with chronic hepatitis C: correlation with serum hepatitis C virus-RNA levels and response to treatment with interferon. *J. Gastroenterol. Hepatol. 12:460.*
17. Morita, T., K. Tanaka, S. Saito, T. Kitamura, M. Kondo, T. Sakaguchi, M. Marimoto, and H. Sekihara. 1998. Expression of interferon receptor genes (IFN-α-R1 and IFN-α-R2 mRNA) in the liver may predict outcome after interferon therapy in patients with chronic genotype 2a or 2b hepatitis C virus infection. *J. Clin. Gastroenterol. 26:135.*
18. Antonelli, G., and F. Dianzani. 1999. Development of antibodies to interferon beta in patients: technical and biological aspects. *Eur. Cytokine. Rev. 10:413.*
19. Shindo, M., G. Mullin, L. Braun-Elwert, N. Bergasa, E. A. Jones, and S. P. James. 1996. Cytokine mRNA expression in the liver of patients with primary biliary cirrhosis (PBC) and chronic hepatitis B (CHB). *Clin. Exp. Immunol. 105:254.*
20. Llorent. L., Y. Richaud-Patin, N. Alcocer-Castillejos, R. Ruiz-Soto, M. Mercado, H. Orozco, A. Gamboa-Dominguez, and J. Alcocer-Varela. 1996. Cytokine gene expression in cirrhotic and non-cirrhotic human liver. *J. Hepatol. 24:555.*
21. Mazzella, G., G. Saracco, D. Festi, F. Rosina, S. Marchetto, F. Jaboli, R. Sostegni, A. Pezzoli, F. Azzaroli, C. Cancellieri, et al. 1999. Long-term results with interferon therapy in chronic type B hepatitis: a prospective randomized trial. *Am. J. Gastroenterol. 94:2246.*
22. von Schweinitz, D., M. Hadam, K. Welte, H. Mildenberger, and T. Pietsch. 1993. Production of interleukin-1β and interleukin-6 in hepatoblastoma. *Int. J. Cancer 12:728.*
23. Diehl, A. M. 1999. Cytokines and the molecular mechanisms of alcoholic liver disease. *Alcohol Clin. Exp. Res. 23:1419.*
24. McClain, C., D. Hill, J. Schmidt, and A. M. Diehl. 1993. Cytokines and alcoholic liver disease. *Semin. Liver Dis. 13:170.*
25. McClain, C., S. Barve, I. Deaciuc, M. Kugelmas, and D. Hill. 1999. Cytokines in alcoholic liver disease. *Semin. Liver Dis. 19:205.*
26. Le Moine, O., A. Marchant, M. Goldman, E. Dupont, and J. Deviere. 1994. Cytokines in alcoholic liver cirrhosis. *Acta Gastroenterol. Belg. 57:245.*
27. Diez, R. A., P. Santos, M. G. Lopez, C. J. Gonzalez, E. B. Gil, and G. F. Gutierrez. 1993. Tumour necrosis factor, interleukin-1 and interleukin-6 in alcoholic cirrhosis. *Alcohol 28:319.*
28. Dinarello, C. A. 1997. Interleukin-1. *Cytokine Growth Factor Rev. 8:253.*
29. Thanos, D., and T. Maniatis. 1995. NF-κB: a lesson in family values. *Cell 80: 529.*
30. Verma, I. M., and J. Stevenson. 1997. IκB kinase: beginning, not the end. *Proc. Natl. Acad. Sci. USA 94:11758.*
31. Schulze-Osthoff, K., D. Ferrari, K. Riehemann, and S. Wesselborg. 1997. Regulation of NF-κB activation by MAP kinase cascades. *Immunobiology 198:35.*
32. Stark, G. R., I. M. Kerr, R. B. Williams, R. H. Silverman, and R. D. Schreiber. 1998. How cells respond to interferons. *Annu. Rev. Biochem. 67:227.*
33. Platanias, L. C., and E. N. Fish. 1999. Signaling pathways activated by interferons. *Exp. Hematol. 27:1583.*
34. Haque, S. J., and B. R. Williams. 1998. Signal transduction in the interferon system. *Semin. Oncol. 25:14.*
35. Larner, A., and N. C. Reich. 1996. Interferon signal transduction. *Biotherapy 8:175.*
36. Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell 84:443.*
37. Meraz, M. A., J. M. Whitem, K. C. Sheehan, E. Bach, S. J. Rodig, A. Dighe, D. Kaplan, J. Riley, A. Greenlund, D. Campbell, et al. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell 84:431.*
38. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65:55.*
39. Chen. J., H. Bao, S. Sawyer, G. Kunos, and B. Gao. 1997. Effects of short and long term ethanol on the activation of STAT3 in normal and regenerating liver. *Biochem. Biophys. Res. Commun. 239:666.*
40. Gao, B., and G. Kunos. 1994. Transcription of the rat α1B adrenergic receptor gene in liver is controlled by three promoters. *J. Biol. Chem. 269:15762.*
41. Aebi, M., J. Fah, N. Hurt, C. Samuel, D. Thomis, L. Bazzigher, J. Pavlovic, O. Haller, and P. Staeheli. 1989. cDNA structures and regulation of two interferon-induced human Mx proteins. *Mol. Cell. Biol. 9:5062.*
42. Knook, D. L., and C. H. Sleyster. 1976. Separation of Kupffer and endothelial cells of the rat liver by centrifugal elutriation. *Exp. Cell. Res. 99:444.*
43. Allgood, V. E., Y. Zhang, B. W. O'Malley, and Weigel, N. L. 1997. Analysis of chicken progesterone receptor function and phosphorylation using an adenovirus-mediated procedure for high-efficiency DNA transfer. *Biochemistry 36:224.*
44. Nguyen, V., and Gao, B. 1999. Cross-talk between α(1B)-adrenergic receptor (α(1B)AR) and interleukin-6 (IL-6) signaling pathways. Activation of α(1b)AR inhibits il-6-activated STAT3 in hepatic cells by a p42/44 mitogen-activated protein kinase-dependent mechanism. *J. Biol. Chem. 274:35492.*
45. Tsukamoto, H., and M. Lin. 1997. The role of Kupffer cells in liver injury. In *Cells of the Hepatic Sinusoid*, Vol. 6. E. Wisse, D. L. Knook, and C. Balabaud, eds. The Kupffer Cell Foundation, Leiden, p. 244.
46. Koop, D. R., B. Klopfensetin, Y. J. Iimuro, and R. G. Thurman. 1997. Gadolinium chloride blocks alcohol-dependent liver toxicity in rats treated chronically with intragastric alcohol despite the induction of CYP2E1. *Mol. Pharmacol. 51: 944.*
47. Adachi, Y., B. Bradford, W. Gao, H. Bojes, and R. G. Thurman. 1994. Inactivation of Kupffer cells prevents early alcohol-induced liver injury. *Hepatology 20:453.*
48. Holye, G. W., and R. L. Hill. 1991. Structure of the gene for a carbohydrate-binding receptor unique to rat Kupffer cells. *J. Biol. Chem. 266:1850.*
49. Oda, K., T. Fujiwara, and Y. Ikehara. 1990. Brefeldin A arrests the intracellular transport of viral envelope proteins in primary cultured rat hepatocytes and HepG2 cells. *Biochem. J. 265:161.*
50. Takahara, T., Y. Fukuyama, S. Saito, T. Ogino, N. Miyajima, and M. Kohase. 1999. IL-1, EGF, and HGF suppress the antiviral activity of interferon in primary monkey hepatic parenchymal cells. *Jpn. J. Infect. Dis. 52:45.*
51. Kohase, M., Y. Zhang, J. Lin, S. Yamazaki, P. B. Sehgal, and J. Vilcek. 1988. Interleukin-1 can inhibit interferon-β synthesis and its antiviral action: comparison with tumor necrosis factor. *J. Interferon Res. 8:559.*
52. Keskinen, P., M. Nyqvist, T. Sareneva, J. Pirhonen, K. Melen, and I. Julkunen. 1999. Impaired antiviral response in human hepatoma cells. *Virology 263:364.*
53. Kim, T. K., and T. Maniatis. 1996. Regulation of interferon-γ-activated STAT1 by the ubiquitin-proteasome pathway. *Science 273:1717.*
54. Stoiber, D., P. Kovarik, S. Cohney, J. Johnston, P. Steinlein, and T. Decker. 1999. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-γ. *J. Immunol. 1635:2640.*
55. Boisclair, Y. R., J. Wang, J. Shi, K. Hurst, and G. T. Ooi. 2000. Role of the suppressor of cytokine signaling-3 in mediating the inhibitory effects of interleukin-1β on the growth hormone-dependent transcription of the acid-labile subunit gene in liver cells. *J. Biol. Chem. 275:3841.*
56. Yu, C. L., and S. J. Burakoff. 1997. Involvement of proteasomes in regulating JAK-STAT pathway upon interleukin-2 stimulation. *J. Biol. Chem. 272:14017.*
57. Callus, B. A., and B. Mathey-Prevot. 1998. Interleukin-3-induced activation of the JAK-STAT pathway is prolonged by proteasome inhibitors. *Blood 9:3182.*
58. Gebert, C. A., S. Park, and D. J. Waxman. 1999. Termination of growth hormone pulse-induced STAT5b signaling. *Mol. Endocrinol. 13:38.*
59. Starr, R., and D. J. Hilton. 1999. Negative regulation of the JAK/STAT pathway. *BioEssays 21:47.*